

*Research article***INCREASED ACTIVITY OF HIPPOCAMPAL ANTIOXIDANT ENZYMES AS AN IMPORTANT ADAPTIVE PHENOMENON OF THE ANTIOXIDANT DEFENSE SYSTEM IN CHRONICALLY STRESSED RATS**

POPOVIĆ Nataša, PAJOVIĆ B. Snežana, STOJILJKOVIĆ Vesna,  
TODOROVIĆ Ana, PEJIĆ Snežana, PAVLOVIĆ Ivan, GAVRILOVIĆ Ljubica\*

Institute of Nuclear Sciences “Vinča”, Laboratory of Molecular Biology and Endocrinology, University of Belgrade, Belgrade, Serbia

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This study examined the effects of chronic restraint stress (CRS: 2 hours  $\times$  14 days) on gene expression of three antioxidant enzymes, copper, zinc superoxide dismutase (SOD 1), manganese superoxide dismutase (SOD 2) and catalase (CAT) in the rat hippocampus. Also, we examined changes in the activities of SOD 1, SOD 2 and CAT in the hippocampus of chronically stressed rats. Investigated parameters were quantified by using real-time RT-PCR, Western blot analysis and assay of enzymatic activity.

We found that CRS did not change mRNA and protein levels of SOD 1 and CAT, but increased mRNA and protein levels of SOD 2. However, CRS treatment increased the enzyme activities of SOD 1, SOD 2 and CAT.

Our findings indicate that the increased activity of antioxidant enzymes (SOD 1, SOD 2 and CAT) in the hippocampus may be an important adaptive phenomenon of the antioxidant defense system in chronically stressed rats.

**Keywords:** chronic restraint stress, copper-zinc superoxide dismutase, manganese superoxide dismutase, catalase, hippocampus, rats

## INTRODUCTION

The hippocampal formation of the brain is involved in episodic, declarative, spatial, and contextual learning and memory and is also a particularly vulnerable and sensitive region of the brain to stress [1]. It is well known that intensive stress response results in the production of reactive oxygen species (ROS), i.e. superoxide anion radical, hydroxyl radical and hydrogen peroxide that cause lipid peroxidation, especially in the membranes and can play an important role in tissue injury [2]. For example, immobilization stress generated oxidative stress in the rat brain [3]. Increased ROS levels in the *central nervous system* (CNS) have been associated with the development of

\*Corresponding author: e-mail: gljubica@vin.bg.ac.rs

a number of neuropsychiatric diseases [4]. Oxidative stress plays a critical role in the degeneration of dopamine neurons in Parkinson's disease [5]. Masood et al. [6] reported direct involvement of oxidative stress in anxiety-like behavior in rodents. The first line of defense includes antioxidant enzymes such as copper, zinc superoxide dismutase (SOD 1), manganese superoxide dismutase (SOD 2) and catalase (CAT) which directly remove ROS. It is known that a number of diseases and pathological conditions are related to the long-term adaptive response to stress, particularly under conditions of chronic stress when allostasis can shift from a healthy toward a pathological state [7]. Very little is known about hippocampal gene expression of antioxidant enzymes and the adaptive response antioxidant defense system to chronic stress. Because of the critical role oxidative stress in the development of a number of neuropsychiatric diseases in this study we wanted to investigate whether chronic restraint stress (CRS) changed gene expression of antioxidant enzymes in the rat hippocampus. We applied CRS because Levinstein and Samuels [8] found that it is an effective model for obtaining depressive-like symptoms in rodents. In addition, previous reports showed that CRS can exacerbate neurodegeneration and cognitive deficits [9,10,11].

In this work we investigated how CRS affects the gene expression of three antioxidant enzymes, SOD 1, SOD 2 and CAT in the rats' hippocampus. Also, we examined changes in the activities of SOD 1, SOD 2 and CAT in the hippocampus of chronically stressed rats.

Understanding regulatory molecular mechanisms by which CRS changes antioxidant defense system in the hippocampus in conditions provoked by chronic stress is very important in the research of the adaptive phenomenon of the antioxidant defense system.

## MATERIALS AND METHODS

### Animals and stress models

In this study Wistar male rats (11-week-old) were used. Animals were under standard laboratory conditions with water and food *ad libitum* and kept three to four per cage. Care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee of the Vinča Institute of Nuclear Sciences (No. 01/12 May 28, 2012), Belgrade, Serbia, which follows the guidelines of the registered "Serbian Society for the Use of Animals in Research and Education". Animals were divided into two groups: **CONTROL group** (n=10) was not exposed to any treatment and **CRS group** (n=10) consisted of animals exposed to treatment of chronic restraint stress. Restraint stress was performed by placing each animal in a 25 x 7 cm plastic bottle as described previously [12]. Animals in these groups were exposed to 2h of restraint stress every day at random times during the light period of the light/dark cycle to avoid habituation during the experimental procedure of 14 days [13]. To reduce variance in the physiological parameters due to daily rhythms,

animals were sacrificed at the same time point in the circadian cycle, between 9:00 and 11:00 am, i.e., one day after the last treatment. Animals were sacrificed under no-stress conditions by rapid decapitation. The hippocampus was rapidly dissected, frozen in liquid nitrogen and stored at -70° C until analyzed.

### **RNA isolation and cDNA synthesis**

Methods of RNA isolation and cDNA synthesis is described previously by Gavrilović et al. [14]. Total RNAs were isolated from hippocampal tissue by using TRIZOL reagent (Invitrogen, USA). After the isolation of mRNA, DNA-ase treatment was applied with DNase I (Fermentas, Lithuania). Concentration of total mRNA was measured in triplicates on a spectrophotometer (Pharmacia, GeneQuantII BioTech, USA). Quality of mRNA was checked on agarose gel. Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (Amersham Biosciences, UK) and pd (N)<sub>6</sub> Random Hexamer (Amersham Biosciences, UK) primer according to the manufacturer's protocol. 12 µl of sample which contained 1500 ng mRNA was incubated 10 min at 65°C. Then a 21 µl reverse transcriptase with pd (N) 6 primer (final 0.2 µg) was added per sample and incubated 1 hour at 37°C.

### **Real-time RT-PCR**

CuZn SOD (SOD 1), Mn SOD (SOD 2) and CAT mRNA levels were quantified by quantitative real-time RT-PCR, as described previously by Gavrilović et al. [14]. TaqMan PCR assays were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, USA) for SOD 1 (Rn00566938\_m1), SOD 2 (Rn00690587\_g1) and CAT (Rn00560930\_m1). The gene expression assays contained primers for amplification of the target gene and the TaqMan MGB (Minor Groove Binder) probe 6-FAM dye-labeled for the quantification. Reactions were performed in a 25 µL reaction mixture containing 1x TaqMan Universal Master Mix with AmpErase UNG, 1x Assay Mix (Applied Biosystems, USA) and cDNA template (10 ng of RNA converted to cDNA). PCR was carried out in the ABI Prism 7000 Sequence Detection System at 50°C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The experimental threshold was calculated based on the mean baseline fluorescence signal from cycle 3 to 15 plus 10 standard deviations. The point at which the amplification plot crosses this threshold defined as Ct, represents the cycle number at this point and it is inversely proportional to the number of target copies present in the initial sample. Each sample was run in triplicate and the mean value of each Ct triplicate was used for further calculations. The reference gene (endogenous control) was included in each analysis to correct for the differences in the inter-assay amplification efficiency and all transcripts were normalized to cyclophyline A (Rn00690933\_m1) expression. The reaction mixture for endogenous control gene amplification consisted of 1x TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems, USA), 1x Assay (6-FAM dye-labeled MGB probes) and cDNA

(10 ng of RNA converted to cDNA). The levels of expression of cyclophyline A in samples under different treatments were checked by additional experiments that confirmed that the chosen reference gene was not regulated. Before quantification, validation experiments were performed to determine the similar amplification efficiency of endogenous control and each target gene. We tested cyclophyline A and demonstrated that its efficiency of amplification was approximately equal to all assays used for target genes. Briefly, serial dilutions of cDNA were prepared and amplified by real-time PCR using specific primers and fluorogenic probes for target and endogenous control gene. Quantification was done using the  $2^{-\Delta\Delta C_t}$  method according to Livak and Schmittgen [15]. The results obtained were analyzed by the RQ Study Add On software for 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System, Applied Biosystems, USA) with a confidence level of 95% ( $P < 0.05$ ). The relative expression of the target gene was normalized to cyclophyline A and expressed in relation to the calibrator, i.e. the control sample. Due to individual differences among animals, one sample from the control group with the expression value closest to the mean of all samples in this group and with the lowest measurement error was chosen as a calibrator. The results are reported as a fold change relative to the calibrator and normalized to cyclophyline A using the equation:  $N_{\text{sample}} = 2^{-\Delta\Delta C_t}$ .

### Hippocampal tissue homogenization and measurement of protein concentration

The hippocampus was homogenized in 0.05 M sodium phosphate buffer (pH 6.65). Subsequently, the protein concentration was determined using BCA method (Thermo Scientific Pierce, USA), described by Stich [16].

### Western blot analysis

CuZn SOD (SOD 1), Mn SOD (SOD 2) and CAT proteins were assayed by Western blot analysis as described previously by Gavrilović *et al.* [14]. The samples were boiled in denaturing buffer according to Laemmli [17], for 5 min at 95°C. Fifteen micrograms of protein extract from hippocampus was separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a supported nitrocellulose membrane (Hybond™ C Extra, Amersham Biosciences, UK). The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline-Tween (TBST). All following washes and antibody incubations were also carried out in TBS-T at room temperature on a shaker. Protein molecular mass standards (PageRuler™ Plus Prestained Protein Ladder, Fermentas) were used for calibration. Antibodies used for quantification of specific proteins were as follows: for SOD 1 (SOD-101, Stressgen, USA), for SOD 2 (SOD-110, Stressgen, USA), for CAT (Calbiochem, Germany) and for  $\beta$ -actin (ab8227, Abcam, USA). After washing, the membranes were incubated in the secondary anti-mouse and anti-rabbit (dilution 1:5000, Amersham ECL™ Western Blotting Analysis System, UK) antibodies conjugated to horseradish peroxidase. A secondary antibody was then visualized by the Western blotting enhanced chemiluminiscent detection

system (ECL, Amersham Biosciences, UK). The membranes were exposed to ECL film (Amersham Biosciences, UK). Densitometry of protein bands on ECL film was performed by Image J analysis PC software. The result was expressed in arbitrary units normalized in relation to  $\beta$  actin.

### **Antioxidant enzyme activities**

SOD 1, SOD 2 and CAT activity was determined using previously described methods by Stojiljković et al. [18].

### **Assay of SOD activity**

Total SOD activity was measured using the Oxis Bioxytech® SOD-525™ Assay (Oxis International, Inc., Portland, OR, USA). The method is based on the SOD-mediated increase in the rate of autoxidation of reagent 1 (5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c] fluorene, R1) in aqueous alkaline solution, yielding a chromophore with maximum absorbance at 525 nm. The kinetic measurement of the change in absorbance at 525 nm is performed. One SOD-525 activity unit is defined as the activity that doubles the autoxidation rate of the control blank. CuZnSOD activity was measured as described above, after pretreating samples with ethanol–chloroform reagent (5/3 vol/vol), which inactivates MnSOD. MnSOD activity was then calculated by subtracting CuZnSOD activity from total SOD activity.

### **Assay of CAT activity**

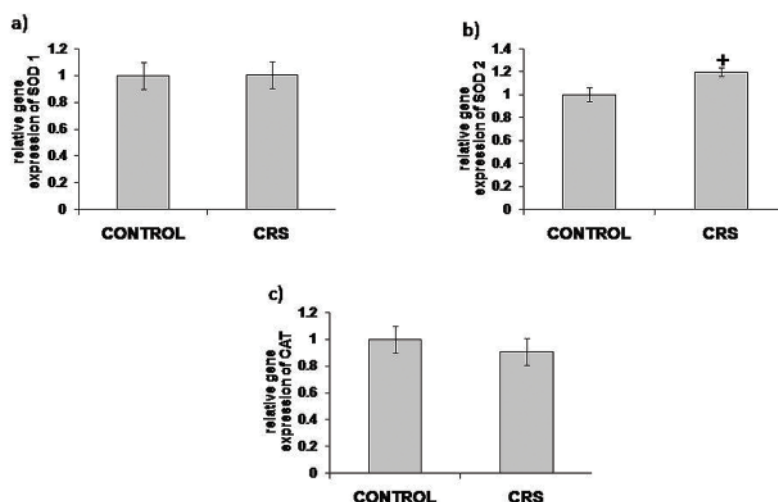
CAT activity was determined by the method of Beutler [19]. The reaction is based on the rate of  $\text{H}_2\text{O}_2$  degradation by catalase contained in the examined samples. The reaction was performed in an incubation mixture containing 1 M Tris-HCl, 5 mM EDTA, pH 8.0, and monitored spectrophotometrically at 230 nm. One unit of CAT activity is defined as 1  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  decomposed per minute under the assay conditions.

### **Data analysis**

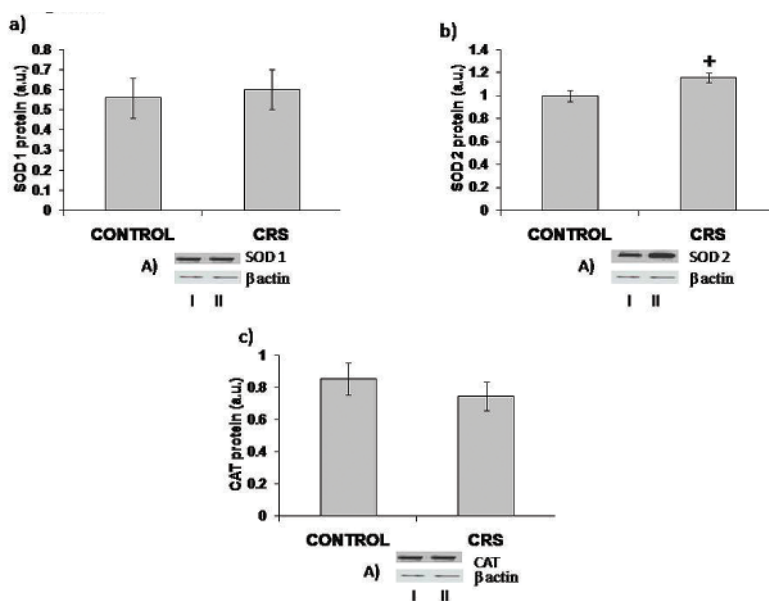
The data are presented as means  $\pm$  S.E.M. Differences of gene expression (mRNA and protein levels) of SOD 1, SOD 2 and CAT, as well as activity of enzymes (SOD 1, SOD 2 and CAT) between control and CRS animals in the hippocampus were analyzed by t-test. Statistical significance was accepted at  $p < 0.05$ .

## **RESULTS**

The animals exposed to CRS showed unchanged levels mRNA and protein of SOD 1 (Fig. 1a and 2a) and CAT (Fig. 1c and 2c), as well as significantly increased levels of SOD 2 mRNA by 19% ( $p < 0.05$ , t-test, Fig. 1b) and SOD 2 protein level of by 15% ( $p < 0.05$ , t-test, Fig.2b) compared with control animals.



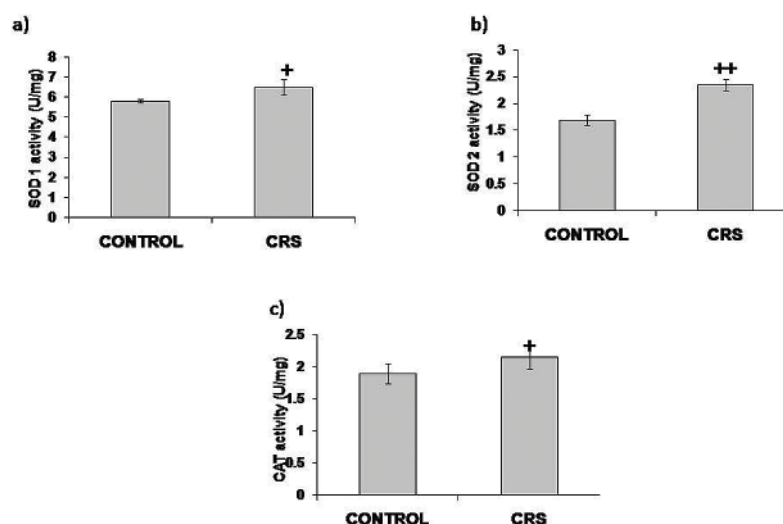
**Figure 1.** Effects of chronic restraint stress (CRS) on CuZn superoxide dismutase (SOD1) [a], Mn superoxide dismutase (SOD2) [b] and catalase (CAT) [c] mRNA levels in the hippocampus. The values are means  $\pm$  S.E.M. of 10 rats. Statistical significance: + $p < 0.05$  animals exposed to chronic restraint stress vs. control animals (t-test). The final result was expressed as fold change relative to the calibrator and normalized to cyclophyline A.



**Figure 2.** Effects of chronic restraint stress (CRS) on CuZn superoxide dismutase (SOD1) [a], Mn superoxide dismutase (SOD2) [b] and catalase (CAT) [c] protein levels in the hippocampus. The values are means  $\pm$  S.E.M. of 10 rats. Statistical significance: + $p < 0.05$  animals exposed to chronic restraint stress vs. control animals (t-test). The result was expressed in arbitrary units normalized in relation to  $\beta$  actin.

(A) Distribution of SOD1, SOD2, CAT and  $\beta$ -actin proteins in the hippocampus of control animals [I], animals exposed to CRS [II].

CRS treatment significantly increased the enzyme activities of SOD 1 by 12% ( $p < 0.05$ , t-test, Fig. 3a), SOD 2 by 39% ( $p < 0.01$ , t-test, Fig. 3b) and CAT by 14% ( $p < 0.05$ , t-test, Fig. 3c) compared with control animals.



**Figure 3.** Effects of chronic restraint stress (CRS) on CuZn superoxide dismutase (SOD1) [a], Mn superoxide dismutase (SOD2) [b] and catalase (CAT) [c] enzyme activities in the hippocampus. The values are means  $\pm$  S.E.M. of 10 rats. Statistical significance: + $p < 0.05$ , ++ $p < 0.01$  animals exposed to chronic restraint stress *vs.* control animals (t-test). The final result for enzyme activity was expressed as units per milligram of protein (U/mg).

## DISCUSSION

The adaptive response of the antioxidant system, and consequently of the expression of the various indirect markers of oxidative tissue damage, would also appear to be specific, to either the type of tissue or the different antioxidant systems involved [20,21]. In this study we have found that the CRS did not change levels of mRNAs and protein of SOD 1 and CAT in the hippocampus. However, CRS treatment significantly increased the enzyme activities of SOD 1 and CAT. Many factors can affect the enzyme activities without changing their gene expression. For example discrepancies between mRNA levels and enzyme activities may be related to differences in mRNA stability or translational efficiency [22]. Regulation of expression might act on individual mRNAs to block their translation and thereby lead to their degradation [23]. Our results support the reports of García-López et al. [23] that the message degradation may be the primary target of regulation of gene expression in chronic stress conditions. Differences between mRNA levels and activities of SOD may be in a kinase/phosphatase signal transduction pathway that may exert a fine control over post-transcriptional regulation of SOD expression [24]. In addition, CAT may be inactivated by its substrate, hydrogen peroxide, due to formation of complex II or

complex III of CAT at high peroxide concentrations [25]. However, in the present study the animals exposed to CRS showed significantly increased gene expression and enzyme activities of SOD 2. The increased hippocampal SOD 2 transcript levels suggest that chronic stress induces increased readiness of the SOD 2 for possible other stressor that may be encountered later. The increased activity of antioxidant enzymes (SOD 1, SOD 2 and CAT) in the hippocampus was found to be an important adaptive phenomenon of the antioxidant defense system in chronically stressed rats. Our data suggest readiness of antioxidant enzymes (SOD 1, SOD 2 and CAT) in the hippocampus to repair or prevent damage due to reactive oxygen species in chronically stressed animals. The increased activity of SOD during chronic stress is an indicator of a relative increase in the superoxide radical production, which could stimulate the second line of defense including CAT [26]. Treatment with antidepressants significantly decreased the activities of SOD and CAT in depressive patients [27,28,29].

Our findings support the idea that the message degradation (SOD 1 and CAT) and increased activity of antioxidant enzymes (SOD 1, SOD 2 and CAT) in the hippocampus may be an important adaptive phenomenon of the antioxidant defense system in chronically stressed rats.

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### **Authors' contributions**

NP participated in the experiment, carried out the molecular genetic studies. SBP helped to draft the manuscript. VS participated in the experiment, helped to draft the statistical analysis, helped to draft the manuscript. AT participated in the experiment. SP helped to draft the statistical analysis. IP participated in the experiment. GLj participated in the experiment, carried out the molecular genetic studies, participated in the statistical analysis, designed and wrote manuscript. All authors read and approved the final manuscript.

### **Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.



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## **POVEĆANA AKTIVNOST ANTIOKSIDATIVNIH ENZIMA HIPOKAMPUSA JE VAŽAN ADAPTIVNI FENOMEN SISTEMA ANTIOKSIDATIVNE ZAŠTITE HRONIČNO STRESIRANIH PACOVA**

POPOVIĆ Nataša, PAJOVIĆ B. Snežana, STOJILJKOVIĆ Vesna,  
TODOROVIĆ Ana, PEJIĆ Snežana, PAVLOVIĆ Ivan, GAVRILOVIĆ Ljubica

Ova studija ispitala je uticaj hroničnog stresa fizičkog sputavanja (CRS: 2 sata x 14 dana) na ekspresiju gena za tri antioksidativna enzima (bakar-cink superoksid dismutaza-SOD 1, mangan superoksid dismutaza-SOD 2 i katalaza-CAT) u hipokampusu pacova. Takođe, ispitali smo i promene aktivnosti SOD 1, SOD 2 i CAT enzima

u hipokampusu hronično stresiranih pacova. Ispitivani parametri kvantifikovani su korišćenjem RT-PCR-a u realnom vremenu, Western blot analize i eseja za aktivnost enzima.

Pronašli smo da CRS ne menja nivoe iRNK i nivoe proteina SOD 1 i CAT, ali da povećava nivoe iRNK i nivoe proteina SOD 2. Međutim, CRS tretman povećao je aktivnosti SOD 1, SOD 2 i CAT enzima.

Naši nalazi ukazuju da povećana aktivnost enzima (SOD 1, SOD 2 i CAT) u hipokampusu može biti važan adaptivni fenomen sistema antioksidativne zaštite hronično stresiranih pacova.